European Journal of Pharmaceutical Sciences 131 (2019) 159–166

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

A new, vasoactive hybrid aspirin containing nitrogen monoxide-releasing molsidomine moiety

Kitti Szőke[a], Attila Czompa[a], István Lekli[a], Péter Szabados-Fürjesi[a,b], Mihály Herczeg[c], Magdolna Csávás[c], Anikó Borbás[c], Pál Herczegh[c,*], Árpád Tósaki[a,**]

[a] Department of Pharmacology, Faculty of Pharmacy, University of Debrecen, Debrecen, Hungary
[b] Department of Bioanalytical Chemistry, Faculty of Pharmacy, University of Debrecen, Debrecen, Hungary
[c] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Debrecen, Debrecen, Hungary

ARTICLE INFO

Keywords:
Acetylsalicylic acid
Molsidomine
Nitrogen oxide liberation
Isolated heart
Vasoactivity
Coronary flow

ABSTRACT

Ischemic heart conditions are among the main causes of sudden cardiac death worldwide. One of the strategies for avoiding myocardial infarction is the low-dose, prophylactic use of acetylsalicylic acid (ASA), an inhibitor of platelet aggregation. To avoid the gastrointestinal damage, ASA prodrugs bearing nitric oxide (NO)-donating moiety covalently conjugated to ASA have been synthesized and evaluated extensively worldwide. Herein the synthesis of a new hybrid ASA ester covalently attached to the NO donor linsidomine, an active metabolite of molsidomine (MOL) is reported. Cell viability assay and hemolysis tests were performed in H9c2 cells and rat erythrocytes, respectively. Our new compound, the **ERJ-500** not affected negatively the viability of living cells in the concentration range of 100 nM to 100 μM. Using the ex vivo Langendorff method on hearts originated from female rats, compound **ERJ-500** displayed a dose-dependent, outwashable vasodilative effect in coronary arteries. Vasodilation was observed on isolated working heart model as well, with elevated stroke volume in hearts treated with **ERJ-500**. Furthermore, a decreased infarct size was also noticed in ERJ-500 treated hearts after ischemia/reperfusion. Based on these observations it can be expected that our new hybrid ASA may contribute to new pharmacological tool in the therapy of ischemic heart conditions and associated syndromes.

1. Introduction

Acetylsalicylic acid (ASA), also known as aspirin - the oldest non-steroidal anti-inflammatory drug (NSAID) - is extensively used for the treatment of pain and inflammation and because of its antithrombotic properties, it is also commonly used for the prophylaxis against myocardial infarction and stroke. The anti-inflammatory action of ASA is based on the inhibition of cyclooxygenase (COX1 and COX2) enzymes involved in prostaglandin (PG) biosynthesis (Catella-Lawson et al., 2001b). However, ASA is a much more potent inhibitor of COX1 isoenzyme than that of COX2 (Meade et al., 1993). Moreover, ASA irreversibly inhibits COX1 in platelets, consequently resulting in the inhibition of thromboxane A2 biosynthesis (Catella-Lawson and Crofford, 2001). Since thromboxane A2 is a potent platelet aggregator and causes vasoconstriction, this inhibitory process affects the antiplatelet-aggregation property of ASA. Low-dose, long-term prophylactic use of ASA is limited by its strong local irritant effects and gastrotoxicity (Schoen and Vender, 1989; Wolfe et al., 1999) and ulcerative ability.

There is an increasing number of experimental data supporting basic physiological and protective roles of nitrogen monoxide, also called nitric oxide (NO), and nitrogen monoxide-releasing molecules (NMRMs) in injured tissues (Abu-Amara et al., 2012; Garry et al., 2015; Nagasaka et al., 2008; Phillips et al., 2009). The main source of endogenous NO is nitrogen monoxide synthase (NOS). NOS/NO system was proved to play an important role in signaling mechanisms and several physiological processes, including the maintenance of neuronal (Prast and Philippu, 2001), immune (Wink et al., 2011), and cardiovascular functions (Strijdom et al., 2009). Moreover, NO acts as a crucial signaling molecule and an effector mediator to regulate the coronary artery function in the myocardium (Bohlen, 2015). However, overexpression of inducible NOS and its consequence, an extensive increase in endogenous NO production may not be beneficial for the myocardium (Csonka et al., 1999; Varga et al., 1999). On the other hand, molecules releasing NO including molsidomine (MOL) are used as

* Correspondence to: P. Herczegh, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Debrecen, Egyetem tér 1, H-4032 Debrecen, Hungary.
** Correspondence to: Á. Tósaki, Department of Pharmacology, Faculty of Pharmacy, University of Debrecen, Nagyerdei Krt 98, H-4032 Debrecen, Hungary.
E-mail addresses: Herczegh.pal@pharm.unideb.hu (P. Herczegh), tosaki.arpad@pharm.unideb.hu (Á. Tósaki).

https://doi.org/10.1016/j.ejps.2019.02.020
Received 22 February 2018; Received in revised form 13 February 2019; Accepted 14 February 2019
Available online 16 February 2019
0928-0987/ © 2019 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/BY-NC-ND/4.0/).

antihypertensive and antianginal drugs.

The strategy for avoiding the systemic gastrointestinal damage, ASA prodrugs bearing nitrogen monoxide (NO)–donating moiety covalently attached to the carboxylic function of ASA were designed since locally released NO is able to trigger anti-inflammatory effects (MacNaughton et al., 1989; Wallace, 2007). Nitrate ester (Gilmer et al., 2007; Lazzarato et al., 2009; Rolando et al., 2013), furoxan (Cena et al., 2003), or diazenium-diolate derivatives (Abdellatif et al., 2009; Velazquez et al., 2005; Velazquez et al., 2008) have been attached covalently to ASA to form ester-type prodrugs. The biological activity of these hybrid aspirins has been evaluated extensively. Thus, nitrate ester derivative NCX4016 prevented thromboembolism and restenosis and protected the heart from ischemia/reperfusion injury in animal models (Gresele and Momi, 2006) displaying no gastrotoxicity in the stomach. Further beneficial effects include the inhibition of platelet COX1 activation and favorable influence on platelet-activation function in healthy volunteers (Gresele and Momi, 2006). Additional advantageous properties of ASA and NMRMs include anti-inflammatory and gastrosparing activities (Abdellatif et al., 2009; Cena et al., 2003; Lazzarato et al., 2009; Rolando et al., 2013; Velazquez et al., 2005; Velazquez et al., 2008). Furthermore, various NO donors have been developed as pharmacological tools to induce the protective effect of the ischemic myocardium (Ruiz-Hurtado et al., 2007). The NMRMs release NO into biological systems for therapeutic purposes in a controlled and safe manner (Burgaud et al., 2002). The cardiovascular effects of NMRMs are currently under intensive investigation and various classes of compounds are being developed with the goal of exploiting therapeutic potentials in the treatment of inflammatory and cardiovascular diseases (Bell et al., 2003; Ripamonti et al., 2017). Thus, it is quite rational to hypothesize that an NMRM bearing ASA and molsidomine may have vasoactive, and COX inhibitor activity. Acetylsalicylic acid and molsidomine are used as drugs for several decades. Therefore, their side effects and pharmacokinetics are well known. Moreover, molsidomine excels from NO donor compounds since during long-term treatment with it, tolerance development is not a clinically relevant problem (Rudolph and Dirschinger, 1991).

2. Material and methods

2.1. Chemistry

MOL derivative **7** was prepared according to literature procedures (Soulère et al., 2003). All reagents were purchased from commercial suppliers and used without further purification. TLC was performed on Kieselgel 60 F_{254} (Merck, Darmstadt, Germany) with detection by UV-light (254 nm) and immersing into sulfuric acidic ammonium-molibdenate solution followed by heating. Flash column chromatography was performed on Silica gel 60 (Merck 0.040–0.063 mm). Organic solutions were dried over Na_2SO_4 or $MgSO_4$ and concentrated in vacuum. The ^{1}H NMR (400 MHz) and ^{13}C NMR (101 MHz) spectra were recorded with a Bruker DRX-400 spectrometer at 25 °C. Chemical shifts are referenced to Me_4Si (0.00 ppm for ^{1}H) and to the residual solvent signals ($CDCl_3$: 77.1 for ^{13}C). MALDI-TOF MS analyses of the compounds were carried out in the positive reflectron mode using a BIFLEX III mass spectrometer (Bruker, Karlsruhe, Germany) equipped with delayed-ion extraction. 2,5-Dihydroxybenzoic acid (DHB) was used as matrix and $F_3CCOONa$ as cationising agent in DMF.

2.1.1. Compound 5

Compound **4** (4.37 g, 10 mmol) was dissolved in dry dichloromethane (50 ml) and Et_3N (2 ml) was added to the stirred solution. Compound **3** (1.99 g, 10 mmol) dissolved in dry dichloromethane (10 ml) was added dropwise at 0 °C to the reaction mixture and it was stirred for 5 h at room temperature. The reaction mixture was quenched with satd. aq. $NaHCO_3$ (30 ml), stirred for further 15 min, then it was diluted with dichloromethane (100 ml) and extracted with 10% $NaHSO_4$ (30 ml) and water (30 ml), dried over Na_2SO_4, filtered and evaporated at 35 °C in vacuum. The crude product was purified by flash column chromatography (*n*-hexane:acetone 7:3) to give **5** as a pale yellow syrup (4.0 g, 67%). R_f 0.34 (*n*-hexane:acetone 7:3);^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03 (dd, J = 7.8 Hz, J = 1.8 Hz, 1H, arom), 7.53 (td, J = 7.8 Hz, J = 1.8 Hz, 1H, arom), 7.47–7.45 (m, 6H, arom), 7.30–7.19 (m, 10H, arom), 7.08 (dd, J = 8.1 Hz, J = 0.8 Hz, 1H), 4.41–4.39 (m, 2H, TEG-CH_2), 3.78–3.76 (m, 2H, TEG-CH_2), 3.70–3.65 (m, 10H, 5 x TEG-CH_2), 3.23 (t, J = 5.2 Hz, 2H, TEG-CH_2), 2.34 (s, 3H, CH_3 Ac); ^{13}C NMR (101 MHz, $CDCl_3$): δ 169.9 (1C, C_q Ac), 164.5 (1C COO), 150.8 (1C, C_q arom), 144.2 (3C, C_q arom), 134.0, 132.0, 128.8, 127.9, 127.0, 126.1, 123.9 (19C, arom), 123.3 (1C, Cq arom), 86.6 (1C, C_q Tr), 70.9, 70.8, 70.7, 69.2, 64.4, 63.4 (8C, 8 x TEG-CH_2), 21.10 (1C, CH_3 Ac); MS (MALDI-TOF): m/z calcd for $C_{36}H_{38}NaO_8$: 621.25 $[M + Na]^+$; found: 621.32.

2.1.2. Compound 6

Compound **5** (1.2 g, 2.0 mmol) was added to the mixture of hexafluoroisopropanol (7.5 ml), $BF_3{\cdot}Et_2O$ (50 µl, 0.2 equiv.) and Et_3SiH (1.2 ml, 3.8 equiv.). After complete conversion of the starting compound (cc. 15 min) the reaction was quenched with satd. aq. $NaHCO_3$ solution (2 ml). The mixture was concentrated in vacuum and the residue was purified by flash column chromatography (*n*-hexane:acetone 1:1) to give compound **6** as a colorless syrup (460 mg, 65%). R_f 0.25 (*n*-hexane:acetone 1:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.05 (dd, J = 7.8 Hz, J = 1.6 Hz, 1H, arom), 7.56 (td, J = 7.9 Hz, J = 1.6 Hz, 1H, arom), 7.32 (td, J = 7.6 Hz, J = 1.2 Hz, 1H, arom), 7.11 (dd, J = 8.1 Hz, J = 1.2 Hz, 1H, arom), 4.45–4.43 (m, 2H, TEG-CH_2), 3.81–3.78 (m, 2H, TEG-CH_2), 3.74–3.65 (m, 10H, 5 x TEG-CH_2), 3.60–3.58 (m, 2H, TEG-CH_2), 2.62 (s, 1H, TEG-O*H*), 2.36 (s, 3H, CH_3 Ac); ^{13}C NMR (101 MHz, $CDCl_3$): δ 169.9 (1C, C_q *C*OO), 164.5 (1C, C_q Ac), 150.8 (1C, C_q arom), 134.1, 132.0, 126.1, 123.9 (4C, arom), 123.2 (1C, C_q arom), 72.5, 70.8, 70.7, 70.6, 70.4, 69.2, 64.3, 61.8 (8C, 8 x TEG-CH_2), 21.1 (1C, CH_3 Ac); MS (MALDI-TOF): m/z calcd for $C_{17}H_{24}NaO_8$: 379.36 $[M + Na]^+$; found: 379.21.

2.1.3. Compound ERJ-500

The starting materials were dried over P_2O_5 overnight. Compound **7** (2.01 g, 6 mmol) was suspended in dry acetonitrile (100 ml) and compound **6** (2.49 g, 7 mmol) dissolved in dry acetonitrile (10 ml) was added. The reaction mixture was stirred at reflux temperature for 2 h, then it was evaporated. The crude product was purified by flash column chromatography (*n*-hexane: acetone 6:4 → 1:1) to give **ERJ-500** as a colorless syrup (758 mg, 41%).

R_f 0.16 (CH_2Cl_2:acetone 8:2); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03 (dd, J = 7.9 Hz, J = 1.7 Hz, 1H, arom), 7.7 s1H, C*H* sydnone, 7.56 (ddd, J = 8.1, 7.4, 1.8 Hz, 1H, arom), 7.31 (td, J = 7.7 Hz, 1.1 Hz, 1H, arom), 7.10 (dd, J = 8.1 Hz, J = 1.1 Hz, 1H, arom), 4.43–4.41 (m, 2H, CH_2 morpholine), 4.26–4.24 (m, 2H, CH_2 morpholine), 3.94–3.92 (m, 4H, 2 x TEG-CH_2), 3.80–3.78 (m, 2H, CH_2 morpholine), 3.74–3.72 (m, 2H, CH_2 morpholine), 3.68–3.63 (m, 8H, 4 x TEG-CH_2), 3.51–3.49 (m, 4H, 2 x TEG-CH_2), 2.35 (s, 3H, CH_3 Ac); ^{13}C NMR (101 MHz, $CDCl_3$): δ 174.2 (1C, C_q carbamate), 169.7 (1C, C_q COO), 164.4 (1C, C_q Ac), 161.2 (1C, C_q sydnone), 150.6 (1C, C_q arom), 133.8, 131.8, 125.9, 123.7 (4C, arom), 123.2 (1C, C_q arom), 70.6, 70.5, 69.3, 69.0, 65.4, 64.6, 64.3, 54.6 (13C, 1 x sydnone-*C*, 4 x morpholine-CH_2, 8 x TEG-CH_2), 20.9 (1C, CH_3 Ac). MS (MALDI-TOF): m/z calcd for $C_{24}H_{32}N_4NaO_{11}$: 575.20 $[M + Na]^+$; found: 575.31.

2.1.4. Oxidation by synthetic porphyrin and the chemical Fenton system

Two reactions were carried out to test the stability of **ERJ-500** molecule under oxidative conditions, based on the method as reported by Csepanyi et al. (Csepanyi et al., 2017) recently, with minor modifications as follows: 50 µl of **ERJ-500** dissolved in acetonitrile was used for synthetic porphyrin oxidation in 10 mM concentration. 400 µl of **ERJ-500** in 2.5 mM concentration for the Fenton reaction. Samples

were drawn at 1 h in the Fenton reactions prior to injecting them instantly to the HPLC and further investigation. Reaction mixtures for blank contained acetonitrile only without **ERJ-500**. The control mixtures contained no peroxide.

2.2. Biological characterization

2.2.1. Determination of cytotoxicity by MTT assay

Assessment of the cytotoxicity of the **ERJ-500,** ASA, and MOL on cellular survival was accomplished using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay based on the method described by Csepanyi et al. (Csepanyi et al., 2017). Briefly, H9c2 cells were treated with 100 nM, 1 μM, 10 μM, 100 μM of **ERJ-500**, 100 μM of MOL, 100 μM of ASA and 1% H_2O_2 (positive control) containing medium for 24 h on 96 well plates. Then, MTT solution was added to the medium and incubated for 3.5 h at 37 °C. After eliminating the solution from the cells, isopropanol was added and incubated for 0.5 h at 37 °C to dissolve the formazan aggregates. Absorbance was measured at 570 nm and 690 nm.

2.2.2. Animals

Female Sprague Dawley (SD) rats with an average weight of 248 ± 6 g were used in the present study. Animals were nutrified with standard rodent chow pellets (R/M-Z + H, ssniff Spezialdiäten GmbH, Soest, Germany) ad libitum with free access to water and kept at an ambient temperature of 25 ± 2 °C, with a relative humidity of 55 ± 5%, and a 12-h light-dark cycle. All animals were treated according to the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research, and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication no. 86–23, revised in 1996). Breeding and handling of animals were approved by the Institutional Animal Care and Use Committee of the University of Debrecen, Debrecen, Hungary.

2.2.3. Determination of hemolytic activity

Hemolysis tests were performed as described by Roka et al. (Roka et al., 2015) with some minor modifications. Rat blood samples were collected to K_3EDTA containing vacuum tubes (BD, Plymouth, UK) and were treated with 100 nM, 1 μM, 10 μM, 100 μM **ERJ-500**, 100 μM of MOL and the same concentration of ASA in phosphate buffered saline (PBS). The percentage of hemolysis was expressed as the ratio of hemoglobin in the supernatant of the different chemical solutions related to the hemoglobin concentration after the complete hemolysis of erythrocytes in water.

2.2.4. Langendorff heart preparation and assessment of heart rate and coronary flow

Rats were anesthetized with an intraperitoneal pentobarbital sodium injection (60 mg/kg), with heparin as an anticoagulant (1000 U/kg). Following the induction of deep anesthesia, chest cavities were opened, hearts were excised and placed in ice-cold modified Krebs-Henseleit bicarbonate (KHB) buffer (containing 118 mM NaCl, 5.8 mM KCl, 1.8 mM $CaCl_2$, 25 mM $NaHCO_3$, 0.36 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 5.0 mM glucose). After excision, aortas were cannulated and each heart was perfused with modified KHB buffer at a filling pressure of 100 cm of water, using the "non-working" Langendorff mode for 5 min in order to flush blood out from the myocardium. The setup was assembled with two buffer-chambers at the same constant pressure. The one contained the KHB buffer only, the other contained **ERJ-500** dissolved into the KHB buffer at different concentrations (1 μM, 10 μM, 30 μM, 100 μM). At the end of the washout period, baseline cardiac parameters were registered, including coronary flow (CF) and heart rate (HR), and the inflow was switched to serve the hearts from the chamber containing **ERJ-500** for 10 min. Next, 10 min of washout period, followed by 10 min of adding once more the **ERJ-500** containing buffer. A continuous pressure signal was recorded during the whole experiment with the help of a pressure transducer (ADInstruments, PowerLab, Castle Hill, Australia), which was calibrated before each experiment. HR was calculated from the continuously recorded pressure signal. CF was assessed by the time-collecting of the coronary effluent.

2.2.5. Isolated working heart preparation to assess cardiac parameters and infarct size

To measure cardiac function, isolated working heart preparations were carried out based on a previously described method by Czompa et al. (Czompa et al., 2014) on Sprague Dawley female rats divided into two groups n = 11 in the control group, n = 6 in the treated group. After completing the isolated working heart preparation procedure followed by 10 min washout period, we registered the baseline working heart parameters such as aorta flow (Catella-Lawson et al., 2001a), coronary flow (CF), aortic pressure (AOP), heart rate (HR) and derivated aortic pressure (AOdP/dT). Cardiac output (CO) was calculated by the sum of AF and CF and we got stroke volume (SV) by dividing the CO with HR. In the treated group, ERJ-500 was added to the KHB buffer by a dilution of a previously prepared stock solution, creating a 100 μM concentration of ERJ-500 in the heart inflow. The molecule-containing KHB buffer was presented after the washout and baseline registration period for 5 mins, followed by a 30 min ischemia followed by 90 min reperfusion. Results of AOP, AOdP/dT, CO and AF are included in a Data in Brief article (Szőke et al., 2019).

To determine the degree of the infarcted area in the myocardium, triphenyl tetrazolium chloride (TTC) staining was performed according to a previously presented study by Czompa et al. (Czompa et al., 2018). Briefly, following ischemia and reperfusion, 50 ml of 1% TTC solution was perfused through the myocardium. Then, hearts were frozen, sectioned, digitalized and all heart sections were blotted dry and weighed. Risked and infarcted areas were quantified by an open-source planimetry software Fiji (Schindelin et al., 2012). Percentage of the infarcted area compared to the whole risked area of the myocardium is represented on a bar chart.

2.2.6. Statistical analyses

All data are presented as the average magnitudes of each outcome in a group ± standard error of the mean (Csepanyi et al.). Statistical analysis was performed using *t*-test or one- or two-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test with GraphPad Prism software for Windows (GraphPad Software Inc., La Jolla, CA, USA). Probability values (p) < 0.05 were considered statistically significant.

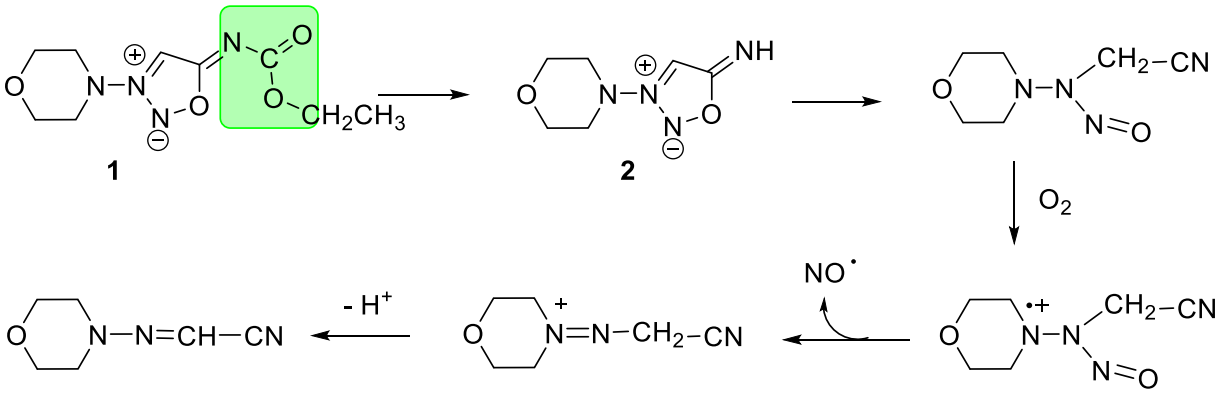
3. Results and discussion

3.1. Chemistry

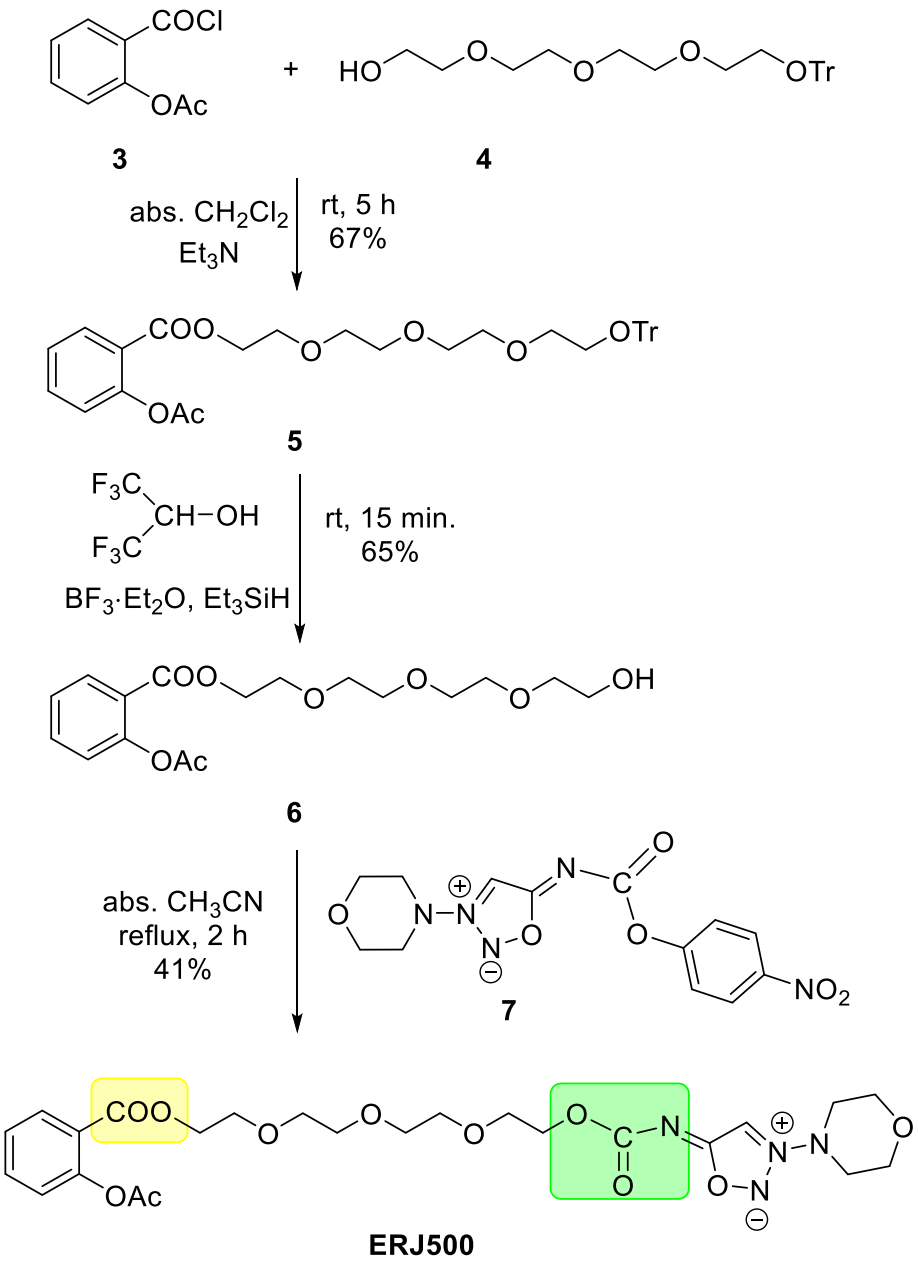
3.1.1. Design and synthesis of ERJ-500

Starting with a research program for the synthesis of new hybrid ASA derivatives we turned our attention to molsidomine (**1**) (3-morpholino-*N*-ethoxycarbonyl sydnonimine), which is a NO donor and used as a coronary vasodilator (Mindlin de Aptecar et al., 1985) in patients suffering from coronary artery diseases. Compound **1** displayed protective effect on indomethacin and ASA-induced gastric injury in rats (Mourad et al., 2000). Moreover, molsidomine (MOL) has a significant platelet antiaggregatory activity in vitro (Nishikawa et al., 1982). We postulated that a hybrid derivative of ASA and MOL would exhibit advantageous and synergistic effects of the two drugs, i.e. diminished side effects of ASA and improved inhibition of platelet aggregation. The mesoionic MOL is metabolized in the following way (Scheme 1) (Reden, 1990).

The active metabolite is **2** (linsidomine, SIN-1), therefore, we hypothesized that its covalent conjugation to acetylsalicylic acid would result in a NO donor hybrid ASA. For the linkage between ASA and



Scheme 1. Metabolism of molsidomine (The carbamate moiety is highlighted in green).



Scheme 2. Synthesis of ASA-molsidomine hybrid with a hydrolysable ester linkage (highlighted in yellow) and the metabolically labile carbamate moiety (highlighted in green).

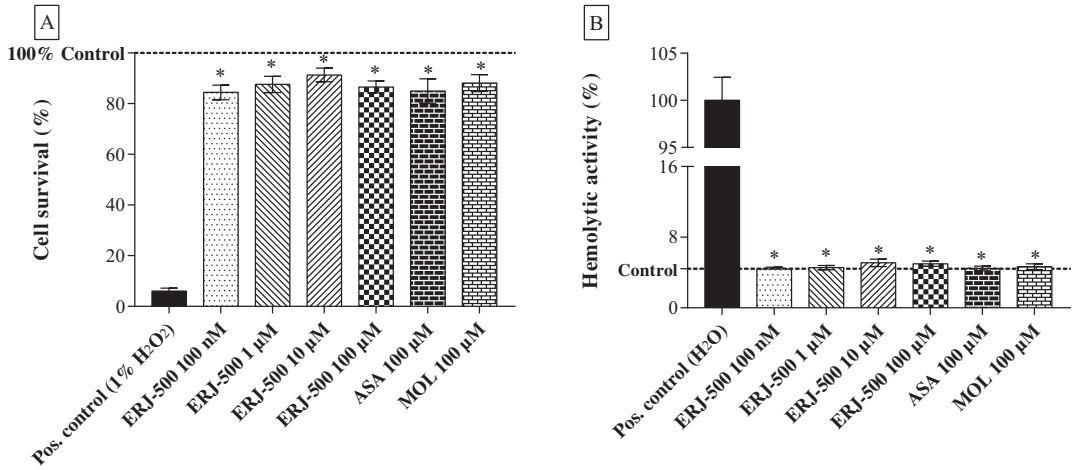


Fig. 1. Safety evaluation of ERJ-500 A. Cytotoxicity test. The bar chart represents cell survival rates in percentage compared to the control group, which served by the solvent only (phosphate buffered saline-PBS). ERJ-500100 nM - 100 µM; ASA 100 µM; MOL 100 µM; and 1% H_2O_2. Results are expressed as mean ± SEM. n = 20–67 cells in each group. *p < 0.05 in comparison with the positive control group (Pos. control).
B. Hemolysis test. The bars represent hemolytic activity in percentage referring to the control group, which contained the solvent (PBS) only. ERJ-500100 nM – 100 µM, ASA 100 µM, MOL 100 µM. Results are expressed as mean ± SEM. n = 8–11 in each group. *p < 0.05 in comparison with the positive control (H_2O) group (Pos. control).

compound **2**, we designed a tetraethyleneglycol chain to improve the water solubility of the product and a carbamate group, similar to that in compound **1**. It is assumed that the planned ASA-MOL conjugate could serve as a NO donor with a similar mechanism to MOL, a drug already used for pharmacotherapy. Our goals practically in the present study were (i) to produce a new NO donor hybrid aspirin and (ii) to study its toxic and vasodilator effects, in the highlight of coronary artery dilation in the myocardium.

For the synthesis of **ERJ-500**, acetylsalicylic acid chloride **3** (Burgstahler et al., 1976) was reacted with mono-triphenylmethyl tetraethyleneglycol **4** (Pilkington-Miksa et al., 2008), obtaining the **5** ester. The trityl group was removed using a reagent cocktail (Kicsak et al., 2016) resulting in compound **6**, which was allowed to react with linsidomine active carbamate ester **7** (Soulère et al., 2003) to give **ERJ-500**, the desired hybrid ASA derivative (Scheme 2).

It is important to note that **ERJ-500** proved to be stable after one-year long storage at room temperature (NMR analysis showed no degradation).

3.1.2. Oxidative stability assays

The oxidative stability of the **ERJ-500** compound was assessed utilizing two novel biomimetic model systems. In the first set of experiments a synthetic porphyrin, Fe(III) meso-tetra(4-sulfonatophenyl) porphine chloride was applied. The total ion chromatograms of the control and **ERJ-500** after oxidation by synthetic porphyrin were almost identical, therefore the **ERJ-500** molecule was resistant against simple oxidative conditions, which could possibly change the structure of the molecule in another case.

The oxidation of the **ERJ-500** was done by the classical Fenton reaction as well. The reaction mixtures were analyzed by HPLC-MS/MS. Based on the recorded spectra of the control and test samples it can be concluded that the compound was stable under the applied conditions, as the peaks on the chromatogram were not changed notably after 1 h of oxidation compared to the control chromatogram. The obtained results were identical to the ones achieved by the synthetic porphyrin oxidation, further confirming the stability of the new molecule under simple oxidative conditions.

A sample chromatogram of each oxidative stability models mentioned above can be found in a Data in Brief article (Szőke et al., 2019).

3.2. Biological studies

3.2.1. Safety evaluation of ERJ-500

To assess the direct cytotoxic effects of **ERJ-500**, we carried out MTT assays at different concentrations of the studied molecule, and its two constituents, ASA and MOL in H9c2 cells. A slight decrement can be seen in all treated groups compared to the control, but all treated groups resulted in a significantly higher cell viability compared to the positive control group, which was treated with 1% H_2O_2. No significant differences can be observed between the groups treated by **ERJ-500** or other molecules studied (Fig. 1.A.), therefore, we may conclude that **ERJ-500** is an equally safe compound as the MOL or ASA.

To confirm our previously demonstrated cytotoxicity results, we performed hemolytic activity studies in blood cells isolated from Sprague Dawley rats. The hemolytic activity in rat erythrocytes at different concentrations of **ERJ-500**, ASA and MOL were significantly lower compared to the positive control group (Fig. 1.B.). Samples of the latter group received sterile water, which induced 100% hemolysis. No significant differences can be observed among the groups treated by **ERJ-500**, ASA, and MOL, respectively in hemolytic activities, which further confirm that our aspirin derivative seems to be a safe compound.

3.2.2. Vasoactive effects of ERJ-500

To study the vasoactive effects of the **ERJ-500** in the myocardium, the drug was dissolved in the perfusion buffer at a concentration rate of 1 µM to 100 µM, and isolated hearts were perfused. During Langendorff perfusion, the **ERJ-500** did not produce any incidence of ventricular tachycardia or ventricular fibrillation. In addition, heart rate was not significantly changed in comparison with the drug-free control group (Fig. 2.A.). Coronary flow was significantly increased by about 50% in the group treated with 100 µM **ERJ-500** (Fig. 2.B.). During Langendorff perfusion, the coronary flow is influenced by the heart rate, the perfusion pressure, and the coronary dimension. Since the perfusion pressure used in the present study is constant and the heart rate is not significantly altered, the increased coronary flow could be a result of the coronary relaxation. Although in the present study, the concentration of NO was not directly measured, and it would be the subject of another study, our results support the hypothesis that NO may originate from the ASA-MOL compound (**ERJ-500**), since salicylic acid shows no vasodilator activity in the myocardium (Andrieu et al., 1999; Saito

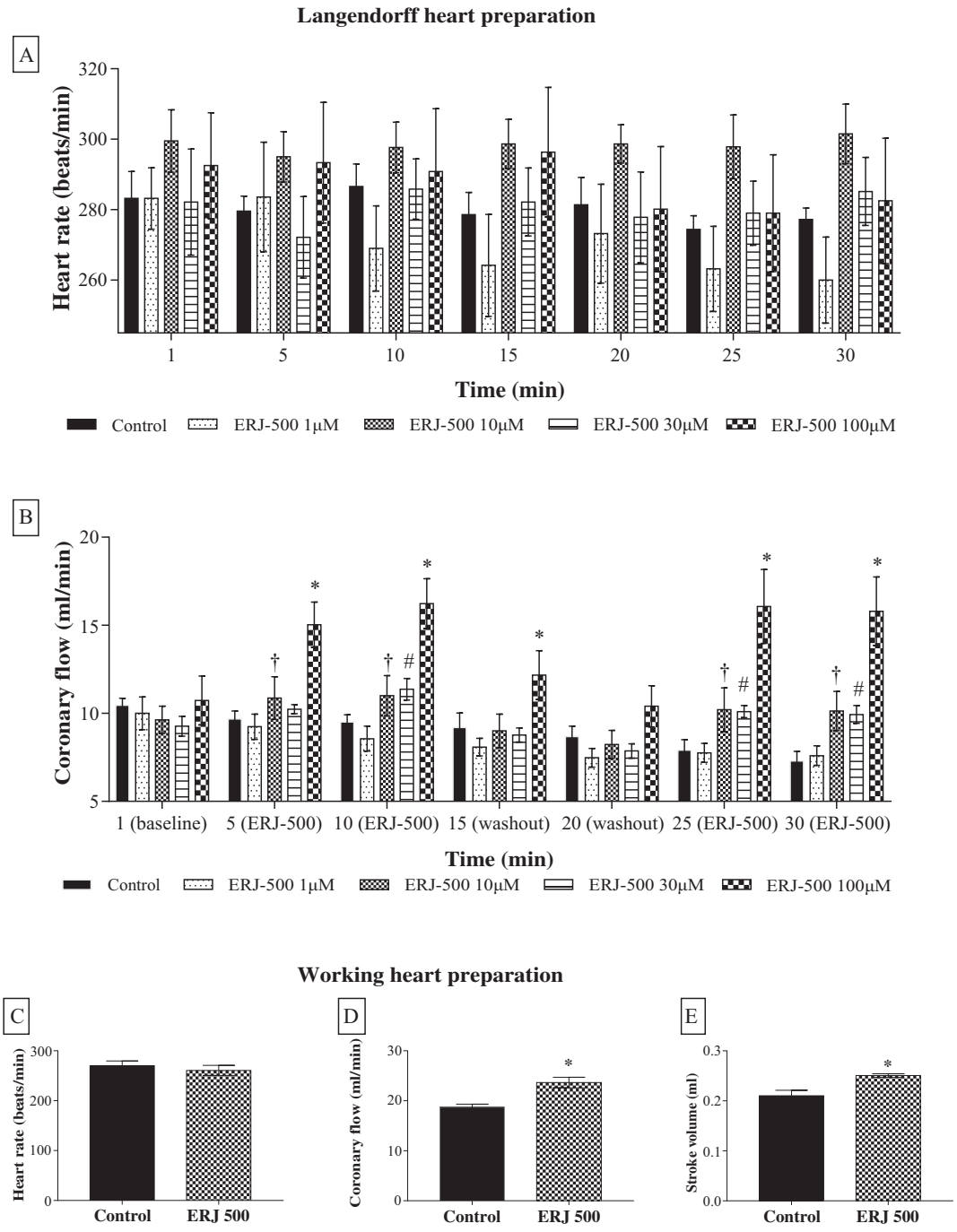


Fig. 2. Effects of ERJ-500 on cardiac functions of isolated Langendorff and working heart.
A., Alteration of heart rate and **B.**, coronary flow in the presence of the ERJ-500 at different concentrations (1–100 μM) when the heart is mounted on the "Langendorff" apparatus. *p < 0.05 control vs. 100 μM; #p < 0.05 control vs. 30 μM; †p < 0.05 control vs. 10 μM. n = 5 in each group.
C., Alteration of heart rate, **D.**, coronary flow, and **E.**, stroke volume in the presence (ERJ-500) or the absence (Control) of 100 μM ERJ-500, when the heart is mounted on the isolated working heart apparatus. No significant differences were observed among groups. *p < 0.05 in comparison with the control values. n = 11 in the control group, n = 6 in the treated group.

et al., 2004).

Cardioprotective effects of ASA and salicylic acid related derivatives can be attributed to affect the platelet activation related to cyclooxygenase enzyme activities (COX1 and COX2) and heat stress protein expression in the diseased myocardium (Rao and Fareed, 2012; Wu et al., 2015).

To further confirm vasoactive effects of **ERJ-500** and to study any possible beneficial effects of the compound on the mechanical activity of the hearts, we tested the molecule on the isolated working heart perfusion system as well, at a concentration, which seemed the most advantageous previously. In the working heart perfusion, when other mechanisms also involved to compensate measurable vasoactive effects, coronary flow was still significantly elevated in treated hearts with 100 μM **ERJ-500** (Fig. 2.D.). Stroke volume was also significantly increased, thus, **ERJ-500** can be an additive effect to improve myocardial contraction force (Fig. 2.E.). As previously measured in Langendorff heart preparation, heart rate did not change notably in working heart preparation also (Fig. 2.C.). Rest of the measured, non-significant

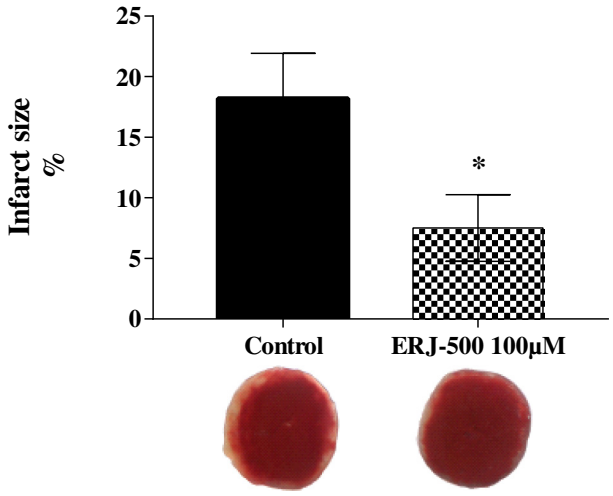


Fig. 3. Effects of ERJ-500 on infarct site. Changes in infarct size after 30 min ischemia followed by 90 min reperfusion, when the hearts are mounted on the isolated working heart apparatus TTC staining method was used. *p < 0.05 in comparison with the control value. n = 5 in each group.

myocardial parameters can be found in a Data in Brief article (Szőke et al., 2019).

3.2.3. *Anti-ischemic effect of ERJ-500*

To further analyze the effect of **ERJ-500** on the rat myocardium, infarct size was evaluated using the triphenyl-tetrazolium-chloride-staining method (TTC). Following 30 min of ischemia and 90 min reperfusion, infarct zones of TTC-stained hearts were expressed in a percentage of the whole myocardium. Fig. 3. shows that hearts perfused with ERJ-500 containing buffer resulted in a significantly decreased infarct size.

This result indicates that ERJ-500 has a cardioprotective effect, which could be a consequence of the vasorelaxant property, however, other mechanisms may also contribute to this effect.

4. Conclusion

In the present study, an attempt was made to synthesize a new NO-releasing ASA derivative and ascertain whether the release of NO from the MOL conjugate could be associated with enhanced myocardial circulation, and consequently, giving a chance to the survival of cardiac cells and tissues by preserving the oxygen supply via the dilation of coronary vessels.

Based on our observations, the new molecule ERJ-500 appears to be nontoxic and stable under oxidative conditions. Furthermore, our pharmacological studies indicate vasoactive and anti-ischemic properties for the molecule. However, further in vivo studies are needed to investigate the effect on whole organism.

5. Limitation of the study

The limitation of the study is the lack of direct measurement of NO release from ERJ-500. However, since in small concentrations of ERJ-500 a significant vasodilatation was observed, the authors believe that it could be the result of NO release from ERJ-500.

Acknowledgement

This study was supported by grants from NKFIH (Nemzeti Kutatási Fejlesztési és Innovációs Hivatal)-124719, Hungary, (A.T.) and OTKA-PD (Országos Tudományos Kutatási Alap - Post. Doc.)-111794, Hungary, (I.L.). This research was also supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4.A/2-11-1-2012-0001, Hungary, (A. Cz., A.T., I.L.) and EFOP-3.6.1-16-2016-00022, Hungary, (K. Sz. and P. Sz.-F.). Supported by the ÚNKP-17-4-III-DE-219 New National Excellence Program of the Ministry of Human Capacities, Hungary (I.L.) and Bolyai Research Scholarship of the Hungarian Academy of Sciences (M. Cs. and M. H.). The authors appreciate the technical assistance for Erzsébet Rőth in the synthesis of **ERJ-500**. A. B and H. P. thank Dr. Georgita Serban for the discussion.

References

Abdellatif, K.R., Chowdhury, M.A., Dong, Y., Das, D., Yu, G., Velazquez, C.A., Suresh, M.R., Knaus, E.E., 2009. Dinitroglyceryl and diazen-1-ium-1,2-diolated nitric oxide donor ester prodrugs of aspirin, indomethacin and ibuprofen: synthesis, biological evaluation and nitric oxide release studies. Bioorg. Med. Chem. Lett. 19, 3014–3018.

Abu-Amara, M., Yang, S.Y., Seifalian, A., Davidson, B., Fuller, B., 2012. The nitric oxide pathway—evidence and mechanisms for protection against liver ischaemia reperfusion injury. Liver Int. 32, 531–543.

Andrieu, S., Lebret, M., Maclouf, J., Beverelli, F., Giudicelli, J.F., Berdeaux, A., 1999. Effects of antiaggregant and antiinflammatory doses of aspirin on coronary hemodynamics and myocardial reactive hyperemia in conscious dogs. J. Cardiovasc. Pharmacol. 33, 264–272.

Bell, R.M., Maddock, H.L., Yellon, D.M., 2003. The cardioprotective and mitochondrial depolarising properties of exogenous nitric oxide in mouse heart. Cardiovasc. Res. 57, 405–415.

Bohlen, H.G., 2015. Nitric oxide and the cardiovascular system. Compr. Physiol. 5, 808–823.

Burgaud, J.L., Ongini, E., Del Soldato, P., 2002. Nitric oxide-releasing drugs: a novel class of effective and safe therapeutic agents. Ann. N. Y. Acad. Sci. 962, 360–371.

Burgstahler, A.W., Weigel, L.O., Shaefer, C.G., 1976. Improved modification of the Rosenmund reduction. Synthesis 1976, 767–768.

Catella-Lawson, F., Crofford, L.J., 2001. Cyclooxygenase inhibition and thrombogenicity. Am. J. Med. 110 (Suppl 3A), 28S–32S.

Catella-Lawson, F., Kapoor, S., Moretti, D., De Marco, S., Vigilante, G.J., Cucchiara, A.J., Ramsey, K.E., Combe, S., Rocca, B., Theroux, P., FitzGerald, G.A., Events, A.I.A.o.t.F.R.a.M., 2001a. Oral glycoprotein IIb/IIIa antagonism in patients with coronary artery disease. Am. J. Cardiol. 88, 236–242.

Catella-Lawson, F., Reilly, M.P., Kapoor, S.C., Cucchiara, A.J., DeMarco, S., Tournier, B., Vyas, S.N., FitzGerald, G.A., 2001b. Cyclooxygenase inhibitors and the antiplatelet effects of aspirin. N. Engl. J. Med. 345, 1809–1817.

Cena, C., Lolli, M.L., Lazzarato, L., Guaita, E., Morini, G., Coruzzi, G., McElroy, S.P., Megson, I.L., Fruttero, R., Gasco, A., 2003. Antiinflammatory, gastrosparing, and antiplatelet properties of new NO-donor esters of aspirin. J. Med. Chem. 46, 747–754.

Csepanyi, E., Szabados-Furjesi, P., Kiss-Szikszai, A., Frensemeier, L.M., Karst, U., Lekli, I., Haines, D.D., Tosaki, A., Bak, I., 2017. Antioxidant properties and oxidative transformation of different chromone derivatives. Molecules 22.

Csonka, C., Szilvassy, Z., Fulop, F., Pali, T., Blasig, I.E., Tosaki, A., Schulz, R., Ferdinandy, P., 1999. Classic preconditioning decreases the harmful accumulation of nitric oxide during ischemia and reperfusion in rat hearts. Circulation 100, 2260–2266.

Czompa, A., Gyongyosi, A., Czegledi, A., Csepanyi, E., Bak, I., Haines, D.D., Tosaki, A., Lekli, I., 2014. Cardioprotection afforded by sour cherry seed kernel: the role of heme oxygenase-1. J. Cardiovasc. Pharmacol. 64, 412–419.

Czompa, A., Szoke, K., Prokisch, J., Gyongyosi, A., Bak, I., Balla, G., Tosaki, A., Lekli, I., 2018. Aged (black) versus raw garlic against ischemia/reperfusion-induced cardiac complications. Int. J. Mol. Sci. 19.

Garry, P.S., Ezra, M., Rowland, M.J., Westbrook, J., Pattinson, K.T., 2015. The role of the nitric oxide pathway in brain injury and its treatment–from bench to bedside. Exp. Neurol. 263, 235–243.

Gilmer, J.F., Moriarty, L.M., Clancy, J.M., 2007. Evaluation of nitrate-substituted pseudocholine esters of aspirin as potential nitro-aspirins. Bioorg. Med. Chem. Lett. 17, 3217–3220.

Gresele, P., Momi, S., 2006. Pharmacologic profile and therapeutic potential of NCX 4016, a nitric oxide-releasing aspirin, for cardiovascular disorders. Cardiovasc. Drug Rev. 24, 148–168.

Kicsak, M., Bege, M., Bereczki, I., Csavas, M., Herczeg, M., Kupihar, Z., Kovacs, L., Borbas, A., Herczegh, P., 2016. A three-component reagent system for rapid and mild removal of O-, N- and S-trityl protecting groups. Org. Biomol. Chem. 14, 3190–3192.

Lazzarato, L., Donnola, M., Rolando, B., Chegaev, K., Marini, E., Cena, C., Di Stilo, A., Fruttero, R., Biondi, S., Ongini, E., Gasco, A., 2009. (Nitrooxyacyloxy)methyl esters of aspirin as novel nitric oxide releasing aspirins. J. Med. Chem. 52, 5058–5068.

MacNaughton, W.K., Cirino, G., Wallace, J.L., 1989. Endothelium-derived relaxing factor (nitric oxide) has protective actions in the stomach. Life Sci. 45, 1869–1876.

Meade, E.A., Smith, W.L., DeWitt, D.L., 1993. Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. J. Biol. Chem. 268, 6610–6614.

Mindlin de Aptecar, F.R., Vazquez, A., Aptecar, M., 1985. Molsidomine–an effective antianginal drug. Results of an acute randomized stress-testing study. Cardiology 72, 185–192.

Mourad, F.H., Khuri, M., Shouaib, F., Nassar, C.F., 2000. Protective effect of the nitric oxide donor molsidomine on indomethacin and aspirin-induced gastric injury in rats. Eur. J. Gastroenterol. Hepatol. 12, 81–84.

Nagasaka, Y., Fernandez, B.O., Garcia-Saura, M.F., Petersen, B., Ichinose, F., Bloch, K.D., Feelisch, M., Zapol, W.M., 2008. Brief periods of nitric oxide inhalation protect against myocardial ischemia-reperfusion injury. Anesthesiology 109, 675–682.
Nishikawa, M., Kanamori, M., Hidaka, H., 1982. Inhibition of platelet aggregation and stimulation of guanylate cyclase by an antianginal agent molsidomine and its metabolites. J. Pharmacol. Exp. Ther. 220, 183–190.
Phillips, L., Toledo, A.H., Lopez-Neblina, F., Anaya-Prado, R., Toledo-Pereyra, L.H., 2009. Nitric oxide mechanism of protection in ischemia and reperfusion injury. J. Investig. Surg. 22, 46–55.
Pilkington-Miksa, M.A., Sarkar, S., Writer, M.J., Barker, S.E., Shamlou, P.A., Hart, S.L., Hailes, H.C., Tabor, A.B., 2008. Synthesis of bifunctional integrin-binding peptides containing PEG spacers of defined length for non-viral gene delivery. Eur. J. Org. Chem. 2008, 2900–2914.
Prast, H., Philippu, A., 2001. Nitric oxide as modulator of neuronal function. Prog. Neurobiol. 64, 51–68.
Rao, G.H., Fareed, J., 2012. Aspirin prophylaxis for the prevention of thrombosis: expectations and limitations. Thrombosis 2012, 104707.
Reden, J., 1990. Molsidomine. Blood Vessels 27, 282–294.
Ripamonti, C., Papagna, A., Storini, C., Miglietta, D., Foti, M., 2017. NO donors exhibit anti-inflammatory properties by modulating inflammatory signatures and by regulating the life cycle of dendritic cells. J. Leukoc. Biol. 102, 1421–1430.
Roka, E., Ujhelyi, Z., Deli, M., Bocsik, A., Fenyvesi, E., Szente, L., Fenyvesi, F., Vecsernyes, M., Varadi, J., Feher, P., Gesztelyi, R., Felix, C., Perret, F., Bacskay, I.K., 2015. Evaluation of the cytotoxicity of alpha-cyclodextrin derivatives on the Caco-2 cell line and human erythrocytes. Molecules 20, 20269–20285.
Rolando, B., Lazzarato, L., Donnola, M., Marini, E., Joseph, S., Morini, G., Pozzoli, C., Fruttero, R., Gasco, A., 2013. Water-soluble nitric-oxide-releasing acetylsalicylic acid (ASA) prodrugs. ChemMedChem 8, 1199–1209.
Rudolph, W., Dirschinger, J., 1991. Clinical comparison of nitrates and sydnonimines. Eur. Heart J. 12 (Suppl E), 33–41.
Ruiz-Hurtado, G., Fernandez-Velasco, M., Mourelle, M., Delgado, C., 2007. LA419, a novel nitric oxide donor, prevents pathological cardiac remodeling in pressure-overloaded rats via endothelial nitric oxide synthase pathway regulation. Hypertension 50, 1049–1056.
Saito, T., Rodger, I.W., Hu, F., Robinson, R., Huynh, T., Giaid, A., 2004. Inhibition of COX pathway in experimental myocardial infarction. J. Mol. Cell. Cardiol. 37, 71–77.
Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682.
Schoen, R.T., Vender, R.J., 1989. Mechanisms of nonsteroidal anti-inflammatory drug-induced gastric damage. Am. J. Med. 86, 449–458.
Soulère, L., Hoffmanna, P., Bringaud, F., 2003. Synthesis of sydnonimine derivatives as potential trypanocidal agents. J. Heterocyclic Chem. 40, 943–947.
Strijdom, H., Chamane, N., Lochner, A., 2009. Nitric oxide in the cardiovascular system: a simple molecule with complex actions. Cardiovasc. J. Afr. 20, 303–310.
Szőke, K., Czompa, A., Lekli, I., Szabados-Fürjesi, P., Herczeg, M., Csávás, M., Borbás, A., Herczegh, P., Tósaki, Á., 2019. Dataset on structure, stability and myocardial effects of a new hybrid aspirin containing nitrogen monoxide-releasing molsidomine moiety. Data Brief (supplement for this article).
Varga, E., Bodi, A., Ferdinandy, P., Droy-Lefaix, M.T., Blasig, I.E., Tosaki, A., 1999. The protective effect of EGb 761 in isolated ischemic/reperfused rat hearts: a link between cardiac function and nitric oxide production. J. Cardiovasc. Pharmacol. 34, 711–717.
Velazquez, C., Praveen Rao, P.N., Knaus, E.E., 2005. Novel nonsteroidal antiinflammatory drugs possessing a nitric oxide donor diazen-1-ium-1,2-diolate moiety: design, synthesis, biological evaluation, and nitric oxide release studies. J. Med. Chem. 48, 4061–4067.
Velazquez, C.A., Chen, Q.H., Citro, M.L., Keefer, L.K., Knaus, E.E., 2008. Second-generation aspirin and indomethacin prodrugs possessing an O(2)-(acetoxymethyl)-1-(2-carboxypyrrolidin-1-yl)diazenium-1,2-diolate nitric oxide donor moiety: design, synthesis, biological evaluation, and nitric oxide release studies. J. Med. Chem. 51, 1954–1961.
Wallace, J.L., 2007. Building a better aspirin: gaseous solutions to a century-old problem. Br. J. Pharmacol. 152, 421–428.
Wink, D.A., Hines, H.B., Cheng, R.Y., Switzer, C.H., Flores-Santana, W., Vitek, M.P., Ridnour, L.A., Colton, C.A., 2011. Nitric oxide and redox mechanisms in the immune response. J. Leukoc. Biol. 89, 873–891.
Wolfe, M.M., Lichtenstein, D.R., Singh, G., 1999. Gastrointestinal toxicity of nonsteroidal antiinflammatory drugs. N. Engl. J. Med. 340, 1888–1899.
Wu, D., Xu, J., Song, E., Tang, S., Zhang, X., Kemper, N., Hartung, J., Bao, E., 2015. Acetyl salicylic acid protected against heat stress damage in chicken myocardial cells and may associate with induced Hsp27 expression. Cell Stress Chaperones 20, 687–696.